IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

The Commissioner of Patents & Trademarks
Washington, D.C. 20231
Attn: Box Patent Application

66/11/80
JC646 U.S. PTO

Docket No. **MERCK 1694 D2**
Prior Application: MERCK 1694 D1
Examiner: A. CAPUTA
Art Unit: 1817

C490 U.S. PTO
09/37/036
08/11/99

Sir: This is a request for filing a
☐ Continuation
☒ Divisional
Of pending prior application Serial No. **08/456,670** filed on **June 1, 1995** of **Peter SCHUBERT, Siegfried NEUMANN; Martina PAWELZIK; Winfried LINXWEILER; Christa BURGER; Gottfried HOFMANN; Andreas BUBERT; Werner GOEBEL; and Stefan KOHLER**, for **PROCESSES AND AGENTS FOR DETECTING LISTERIAS**

1. ☒ Enclosed are 27 pages of the specification including claims and 0 sheets of drawings.

2. ☒ Enclosed is a copy of the oath or declaration as originally filed in Serial No. 08/456,670 on June 1, 1995 in accordance with 37 C.F.R. §1.63(d).

3. ☒ The filing fee is calculated below:

FOR	NUMBER FILED	NUMBER EXTRA	RATE	FEE
TOTAL CLAIMS	38 - 20	18	$18	324.00
INDEPENDENT CLAIMS	6 - 3	3	$78	234.00
☐ MULTIPLE DEPENDENT CLAIM PRESENTED				
☐ Small Entity Status Claimed under 37 CFR 1.9 and 1.27			BASIC FEE	760.00
Statement(s): ☐ Attached ☐ Filed in Parent			TOTAL FILING FEE	$1318.00

4. ☒ The amount of $**1318.00** is included in the attached check.

5. ☒ If a check is not attached, authorization is given to charge the amount indicated in the above sentence to Deposit Account No. 13-3402; two copies of this page being attached for this purpose.

5. ☐ Please charge my Deposit Account No. 13-3402 in the amount of $_____, two copies of this sheet are attached.

6. ☒ The Commissioner is hereby authorized to charge any deficiencies or credit any overpayment in payment of the following fees associated with this communication or otherwise due during the pendency of this application to Deposit Account No. 13-3402.
   ☒ Any filing fees under 37 CFR §1.16 for the presentation of extra claims.
   ☒ Any patent application processing fees under 37 CFR §1.17.

7. ☒ Cancel in this application original claims **1,2 and 11-14** of the prior application before calculating the filing fee.

8. ☒ Amend the specification by inserting before the first line the sentence:
-- This is a ☐ continuation, ☒ division, of prior application Serial No. **08/456,670** filed **June 1, 1995**. --.

9. ☒ Priority of application Nos **4219111.4** filed on **June 11, 1992** in **GERMANY** and **4239567.4** filed on **November 25, 1992** in **GERMANY** is claimed under 35 U.S.C. §119.

10. ☒ The certified copy has been filed in prior application Serial No. **08/456,670** filed **June 1, 1996**.

11. ☒ The prior application is assigned of record to **Merck Patent Gesellschaft Mit Beschrankter Haftung** of **Darmstadt, GERMANY**.

12. ☒ The power of attorney in the prior application is to: I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E.J. Branigan (20,565); John R. Moses (24,983); Harry B Shubin (32,004); Brion P. Heaney (32,542); Diana Hamlet-King (33,302); Richard J. Traverso (30,595); Richard E. Kurtz (33,936); John A. Sopp (33,103); John H. Thomas (33,460); Richard M. Lebovitz (37,067) and Luan C. Do (38,434).
   ☒ a. The power appears in the original papers in the prior application.
   ☒ b. Address all future communications to MILLEN, WHITE, ZELANO & BRANIGAN, P.C.

13. ☒ A preliminary amendment is enclosed.

14. ☐ An Information Disclosure Statement is enclosed.

15. ☒ Incorporation By Reference.
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 2, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

Date:_____

Richard M. Lebovitz (Registration No. 37,067) - Attorney of Record
MILLEN, WHITE, ZELANO & BRANIGAN, P.C.
Arlington Courthouse Plaza I
2200 Clarendon Boulevard, Suite 1400
Arlington, Virginia 22201
(703) 243-6333

K:\PAT\Merck\1694d2\CPA RULE60 FORM.wpd

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	
Peter SCHUBERT et al.	:	Group Art Unit: 1817
Serial No.: NEW	:	Examiner: A. Caputa
Filed: August 11, 1999	:	

For: **PROCESSES AND AGENTS FOR DETECTING LISTERIAS**

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

Prior to examination of the above-identified application, please amend the specification as follows:

IN THE SPECIFICATION:

Page 4,	**line 1:**	After the sequence insert --(SEQ ID NO:1)--;
	line 2:	After the sequence insert --(SEQ ID NO:2)--;
	line 3:	After the sequence insert --(SEQ ID NO:3)--;
	line 4:	After the sequence insert --(SEQ ID NO:4)--;
	line 5:	After the sequence insert --(SEQ ID NO:5)--;
	line 6:	After the sequence insert --(SEQ ID NO:6)--;
	line 7:	After the sequence insert --(SEQ ID NO:7)--;
	line 8:	After the sequence insert --(SEQ ID NO:8)--;
	line 11:	Delete the sequence and insert
		-- ATGAATATGAAAAAAGCAAC (SEQ ID NO:9)--; and
	line 12:	Delete the sequence and insert
		-- TTGGCTTCGGTCGCGTATAA (SEQ ID NO:10)--;
	line 13:	After the sequence insert --(SEQ ID NO:11)--;
	line 14:	After the sequence insert --(SEQ ID NO:12)--;
	line 15:	After the sequence insert --(SEQ ID NO:13)--;

	line 16:	After the sequence insert --(SEQ ID NO:14)--;
	line 17:	After the sequence insert --(SEQ ID NO:15)--;
	line 18:	After the sequence insert --(SEQ ID NO:16)--;
	line 23:	After the sequence insert --(SEQ ID NO:17)--;
	line 24:	After the sequence insert --(SEQ ID NO:18)--;
	line 25:	After the sequence insert --(SEQ ID NO:19)--;
	line 26:	After the sequence insert --(SEQ ID NO:20)--;
	line 27:	After the sequence insert --(SEQ ID NO:21)--;
	line 28:	After the sequence insert --(SEQ ID NO:22)--;
	line 29:	After the sequence insert --(SEQ ID NO:23)--;
	line 30:	After the sequence insert --(SEQ ID NO:24)--;
	line 31:	After the sequence insert --(SEQ ID NO:25)--; and
	line 33:	Change "2a-i," to -- 2a-i (SEQ ID NO:26-34, respectively), --, and Change "5a-d." to -- 5a-d (SEQ ID NO:35-38, respectively).--.
Page 5,	**line 2:**	After "3" insert --(SEQ ID NO:39)--;
	line 17:	After the sequence insert --(SEQ ID NO:17)--;
	line 18:	After the sequence insert --(SEQ ID NO:18)--;
	line 19:	After the sequence insert --(SEQ ID NO:19)--;
	line 20:	After the sequence insert --(SEQ ID NO:20)--;
	line 21:	After the sequence insert --(SEQ ID NO:21)--;
	line 22:	Delete the sequence and insert -- X^3ThrProValValLysGlnGluValLysX^4 (SEQ ID NO:22)--;
	line 23:	Delete the sequence and insert -- X^3ValLysGlnProThrThrGlnGlnThrAlaProX^4 (SEQ ID NO:23) --;
	line 24:	After the sequence insert --(SEQ ID NO:24)--; and
	line 25:	After the sequence insert --(SEQ ID NO:25)--.
Page 6,	**line 31**:	After "sequences" insert --(SEQ ID NO:26-34, respectively)--; and
	line 34:	After "sequence" insert --(SEQ ID NO:39)--.
Page 7,	**line 1**:	After "sequence" insert --(SEQ ID NO:40)--; and
	line 4:	After "sequences" insert --(SEQ ID NO:35-38, respectively)--.

Page 8, **line 5**: Delete "nucleotide" and insert --nucleoside--.

Page 12, **line 7**: After the sequence insert --(SEQ ID NO:41)--.

Page 14, **line 10**: After the sequence insert --(SEQ ID NO:10)--; and
line 11: After the sequence insert --(SEQ ID NO:43)--.

Page 18, **line 32**: After the sequence insert --(SEQ ID NO:42)--; and
line 34: After the sequence insert --(SEQ ID NO:42)--.

Page 19, **line 28**: After the sequence insert --(SEQ ID NO:42)--.

Page 20, **line 13**: After the sequence insert --(SEQ ID NO:42)--;
line 22: After the sequence insert --(SEQ ID NO:42)--;
line 28: Delete "again" and insert --against--; and
line 29: After the sequence insert --(SEQ ID NO:42)--.

After Page 22: Insert the attached new pages 22i-22xxviii.

IN THE CLAIMS:

Please cancel claims 1-2 and 11-14 without prejudice or disclaimer.

Please amend the claims as follows:

Claim 8. (Amended) A process for preparing an antibody [capable of specifically binding] which specifically binds to the p60 protein from pathogenic listerias, comprising

immunizing an experimental animal with an immunogen, wherein said immunogen is a polypeptide of [Figure 3] SEQ ID NO:39 or an immunogenic conjugate which comprises a peptide having a sequence selected from the polypeptide sequence of [Figure 3] SEQ ID NO:39, and

isolating the thus-produced antibody.

Claim 9. (Amended) A process of claim 8, wherein the immunogenic conjugate comprises a 7-24 amino acid peptide having a sequence selected from the polypeptide sequence according to [Figure 3] SEQ ID NO:39.

Claim 10. (Amended) A process of claim 8, wherein the immunogenic conjugate comprises a peptide according to one of the formulae [(IVa)-(IVi) of Figure 3] (IVa)-(IVe) (SEQ ID NO:17 - SEQ ID NO.21) of SEQ ID NO:39.

Claim15. (Amended) An isolated antibody which specifically binds to the p60 protein from pathogenic listerias, wherein said antibody binds an epitope from the sequence of the polypeptide of [Figure 3] SEQ ID NO:39.

Claim 16. (Amended) An isolated antibody of claim 15, wherein said antibody binds an epitope comprising a sequence [of Figures 2a-i.] according to one of the formulae (IVa)-(IVe) (SEQ ID NO:17 - SEQ ID NO:21) of SEQ ID NO:39.

Claim 17. (Amended) An isolated antibody which can be prepared by immunizing an experimental animal with a polypeptide according to [Figure 3] SEQ ID. NO:39, or with an immunogenic conjugate which conjugate comprises a 7-24 amino acid peptide having a sequence selected from the polypeptide sequence of [Figure 3] SEQ ID NO:39, wherein said antibody specifically binds to the p60 protein from pathogenic listerias.

Claim 18. (Amended) An isolated antibody which specifically binds a p60 protein from pathogenic listerias, wherein said antibody binds an epitope which comprises a [sequence] peptide of Figures 5a-d (SEQ ID NO:35 - SEQ ID NO:38).

Claim 19. (Amended) An antibody which specifically binds a p60 protein from pathogenic listerias, and which can be prepared by immunizing an experimental animal with an immunogenic conjugate, wherein the conjugate comprises a peptide of Figures 5a-d (SEQ ID NO:35 - SEQ ID NO:38).

Please add the following new claims:

--24. A process of claim 10, wherein the peptide is SEQ ID NO:20.

25. A process of claim 24, wherein the peptide is SEQ ID NO:29.

26. A process of claim 24, wherein the peptide is SEQ ID NO:42.

27. A process of claim 10, wherein the peptide is SEQ ID NO:17.

28. A process of claim 27, wherein the peptide is SEQ ID NO:30.

29. An isolated antibody of claim 15, which binds to an epitope of a peptide SEQ ID NO:20.

30. An isolated antibody of claim 29, which binds to an epitope of a peptide SEQ ID NO:29.

31. An isolated antibody of claim 29, which binds to an epitope of a peptide SEQ ID NO:42.

32. An isolated antibody of claim 15, which binds to an epitope of a peptide SEQ ID NO:17.

33. An isolated antibody of claim 32, which binds to an epitope of a peptide SEQ ID NO:30.

34. An antibody of claim 17, wherein the peptide is SEQ ID NO:20.

35. An antibody of claim 34, wherein the peptide is SEQ ID NO:29.

36. An antibody of claim 34, wherein the peptide is SEQ ID NO:42.

37. An antibody of claim 17, wherein the peptide is SEQ ID NO:17.

38. An antibody of claim 37, wherein the peptide is SEQ ID NO:30.--

REMARKS

For the convenience of the Examiner, it is noted that this application is a divisional of US 08/456,670, in which claims 1-2 and 11-14, drawn to isolated DNA molecules and methods of using them, have been allowed. Those claims have been cancelled in the instant application. US 08/456,670, in turn, claims priority to US 08/075,248 and 08/412,227, both of which were prosecuted with regard to claims drawn to antibodies and/or methods of making them, and both of which applications are now abandoned.

Please transfer to the instant case the CRF diskette (computer readable copy) and the paper copy of the sequence listing which were filed on November 30, 1998 in the parent case, 08/456,670.

With regard to the amendments to the specification:

The amendment to sequence IVf on page 5 corrects an inadvertent error, in accordance with the correct sequence in original claim 3 (one Val residue was omitted in the sequence in the specification).

The amendment to sequence IVg on page 5 corrects an obvious error of capitalizing the "X" in X^4 in accordance with the rest of the nomenclature.

The amendment on page 20 of "again" to "against" is self-evident in the context of the sentence.

The amendments to the specification and claims concerning SEQ ID NO's are for the purpose of putting the application in compliance with 37 C.F.R. § 1.821-1.825 and conform to the sequence listing .

These amendments to the specification correct obvious inadvertent errors which are supported by other disclosures (*e.g.*, in the claims) or are obvious to a skilled worker; the amendments are fully supported in the specification.

With regard to the amendments to the claims:

These amendments focus the prosecution in the present application on antibodies specific for pathogenic listerias, *i.e.*, *Listeria monocytogenes*. This is not to be construed as acquiescence to any prior art rejections in the ancestor application, 08/075,248 (now abandoned), but rather is being done to expedite prosecution of claims relating to aspects of the present invention. Therefore, the full scope of equivalents applies.

Applicants expressly reserve the right to pursue any cancelled subject matter in a continuing application. The newly added claims recite specific embodiments of the invention and are fully supported in the specification.

Respectfully submitted,

Richard M. Lebovitz (Reg. No. 37,067)
Attorney for Applicants

Nancy J. Axelrod (Reg. No. 44,014)
Patent Agent
MILLEN, WHITE, ZELANO & BRANIGAN, P.C.
Arlington Courthouse Plaza I
2200 Clarendon Boulevard, Suite 1400
Arlington, Virginia 22201
(703) 243-6333

RML/NJA/wks
Filed: **August 11, 1999**
K:\PAT\Merck\1694d2\PreAmd.wpd

PROCESSES AND AGENTS FOR DETECTING LISTERIAS

Background of the Invention

The invention relates to agents and processes for detecting bacteria of the genus *Listeria*, in particular *L. monocytogenes*.

The members of the genus *Listeria* are gram-positive rod-shaped bacteria which occur ubiquitously. Seven different species belong to this genus: *L. monocytogenes, L. ivanovii, L. seeligeri, L. welshimeri, L. innocua, L. murrayi* and *L. grayi*. Of these, only *L. monocytogenes* is pathogenic for humans, endangering in particular the newly born, pregnant women and older persons, as well as patients subjected to immunosuppression. *L. monocytogenes* infections frequently have a fatal outcome.

Contaminated foodstuffs, in which the organism can multiply even at low temperatures around 4°C, are frequently the cause of listeria infections. Thus, various listeria epidemics have been attributed to the consumption of contaminated food, such as, for example, raw milk, cheese or coleslaw. Consequently, rapid detection processes for detecting listerias, in particular in foodstuffs or clinical samples, are urgently required. These processes must additionally be able to distinguish between *L. monocytogenes* and the species which are not pathogenic for humans. Furthermore, it must be possible to detect all variants of *L. monocytogenes*, which is the species which is pathogenic for humans. Recent discussions resulted in the proposal to use *L. innocua* as indicator organism for a potential contamination with *L. monocytogenes*. Therefore, the detection of *L. innocua* would be very useful as well.

Detection of *L. monocytogenes* is effected in a known manner using processes which are based on culturing the microorganisms. The process described in Int. J. Food Microbiol. 4 (1987), 249-256 takes two weeks. A somewhat faster process is recommended by the International Dairy Foundation (IDF); however, it takes at least 6-8 days. Both processes are unsuitable for rapid identification because of the time they take. In addition, both processes are labor-intensive, since nutrient media must be inoculated re-

peatedly in order to obtain single colonies, and since the isolates must subsequently be characterized using biochemical and serological methods of investigation.

While the immunological tests which are currently on the market only take a few hours. they do not permit the important differentiation between different species of listerias. In these processes, also, a two-day pre-enrichment cultivation is required.

A method is described in Appl. Environ. Microbiol. 54 (1988), 2933-2937 in which *L. monocytogenes* is specifically detected using synthetic oligodeoxyribonucleotide probes. However, the probes which are used are not sufficiently specific, since they also react with the species *L. seeligeri*, which is not pathogenic for humans. Prior multiplication of the organisms is required for this process as well: samples of foodstuffs, or their dilutions, are spread on agar plates, and then the inoculated plates are incubated and subsequently investigated by the colony hybridization procedure using a radioactively labelled DNA probe. Detection takes place by autoradiography. This method, too, is labor-intensive and time-consuming.

The DNA sequence of the *iap* (invasion-associated protein) gene of *L. monocytogenes* is described in Infect. Immun. 58, 1943-1950 (1990). This gene encodes a protein which is also known under the designation p60 and which occurs in variants in all *Listeria* species. In *L. monocytogenes* this protein is responsible for the ability to invade animal cells. A polynucleotide (400 bases) having a component sequence from this gene is suitable as a DNA probe for distinguishing *L. monocytogenes* from other organisms.

The polymerase chain reaction (PCR) permits the in vitro amplification of nucleic acids, and prior cultivation is generally not necessary when using this process. In order to start the reaction, short nucleic acid fragments (primers) are required, which primers encompass the section of the genome which is to be amplified. Usually, two primers are required, each of which hybridizes with one nucleic acid strand. One of the primers therefore possesses the complementary sequence to the relevant section of the gene. The choice of these primers determines the specificity of the detection reaction. The use of this process for detecting *L. monocytogenes* is described in Appl. Environmental Microbiology 57, 606-609 (1991), in Letters Appl. Microbiol. 11, 158-162 (1990) and in J. Appl. Bact. 70, 372-379 (1991). More extensive information regarding the details of these processes is available in these publications. The DNA primers bind to the gene for listeriolysin, the listeria hemolysin. The specificity of these primers is at least uncertain. as is evident from comments in J. Appl. Bact. 70: *L. seeligeri* cannot be differentiated with certainty from *L. monocytogenes*. The unambiguous detection of *L. monocytogenes* has thus hitherto not been possible using the PCR technique.

Polyclonal antibodies against *L. monocytogenes* p60 also react with the p60 protein of other, non-pathogenic *Listeria* species. Such antibodies are therefore unsuitable for specifically detecting *L. monocytogenes* by immunological processes. It is possible in principle to purify a polyvalent antiserum of this nature by the specific absorption of interfering antibody fractions: for this purpose, p60 protein from all the other *Listeria* species is covalently bound to carriers. The unwanted antibody fractions can be specifically absorbed; an antiserum then remains which only reacts with p60 protein from *L. monocytogenes*. This method for obtaining an *L. monocytogenes*-specific serum is elaborate: substantial quantities of the polyvalent antiserum are required as starting material, as are, in addition, the p60 iap gene products of the different *Listeria* species. The obtention of monoclonal antibodies against p60 protein would not be associated with this large material requirement; nevertheless, the raising of antibodies against particular epitopes depends on chance: it is first of all necessary to prepare a large number of antibody-producing cell clones, from which suitable clones must then be selected. It has thus far not been possible to obtain antibodies in a targeted manner against epitopes which are specific for *L. monocytogenes*. The same holds true for epitopes which are specific for *L. innocua*.

Summary of the Invention

It is the object of the present invention to provide improved agents and methods for differentiating bacteria of the genus *Listeria*, in particular for detecting bacteria of the species *L. monocytogenes*. In particular, primer sequences which are suitable for the PCR technique are provided according to the invention, as are peptides for the targeted production of specific antibodies which are suitable for the immunological detection of the species *L. monocytogenes* and *L. innocua*.

Upon further study of the specification and appended claims, further objects and advantages of this invention will become apparent to those skilled in the art.

The invention relates to primers, selected from the iap gene, for the amplification of nucleic acids, for example by means of the polymerase chain reaction, characterized in that the primers contain, as a component sequence, at least one sequence according to one of the formulae Ia to Ih and/or an affiliated complementary sequence, it being possible for up to 20 further nucleotide moieties to be bound in front of and/or behind this component sequence. Such primers are suitable for detecting and differentiating bacteria of the genus *Listeria*, including in particular the species *L. monocytogenes*, by means of PCR.

AATATGAAAAAAGC	Ia
GCTTCGGTCGCGTA	Ib
ACAGCTGGGATTGC	Ic
ACTGCTAACACAGCT	Id
TAACAGCAATTCAAG	Ie
CTGAGGTAGCGAGC	If
AGCACTCCAGTTGTTA	Ig
GCAGTTTCTAAACCT	Ih

In this context, primers are particularly preferred which contain a sequence according to one of the formulae IIa to IIh and/or an affiliated complementary sequence.

GTCGACTGAATATGAAAAAAGCAAC	IIa
TTGGCTTCGGTCGCGTAGAATTCATA	IIb
GCTACAGCTGGGATTGCGGT	IIc
CAAACTGCTAACACAGCTACT	IId
CAATAACAGCAATTCAAGTGC	IIe
TAACTGAGGTAGCGAGCGAA	IIf
ACTAGCACTCCAGTTGTTAAAC	IIg
CCAGCAGTTTCTAAACCTGCT	IIh

The invention additionally relates to peptides which contain, as a component sequence, at least one sequence according to one of the formulae IIIa to IIIi, it being possible for in each case up to seven amino acids to be bound by peptide linkages in front of and/or behind this component sequence.

ProValAlaProThrGln	IIIa
ThrGlnAlaThrThrProAla	IIIb
AlaIleLysGlnThrAlaAsnThrAla	IIIc
GlnGlnThrAlaProLysAlaProThr	IIId
ValAsnAsnGluValAlaAlaAlaGluLysThrGlu	IIIe
ThrProValValLysGlnGluValLys	IIIf
ValLysGlnProThrThrGlnGlnThrAlaPro	IIIg
IleLysGlnProThrLysThrValAlaPro	IIIh
GlnGlnThrThrThrLysAlaProThr	IIIi

In this context, peptides are particularly preferred which have a sequence according to one of the Figures 2a-i, and Figures 5a-d.

The invention also relates to the use of one of the said peptides, having a component sequence according to one of the formulae IIIa to IIIi, for preparing immunogenic conjugates. Peptides having a sequence according to one of the Figures 2a-i and of the Figures 5a-d are particularly preferred for this purpose.

The invention also relates to an antibody which binds an epitope which is formed from the polypeptide according to Figure 3 or contains a peptide according to one of the formulae IIIa-IIIi, preferably according to one of the Figures 2a-i and of Figures 5a-d.

The invention further relates to an antibody which can be prepared by immunizing an experimental animal with a polypeptide according to Figure 3 or with an immunogenic conjugate which contains a peptide having 7 to 24 amino acids selected from the polypeptide according to Figure 3.

The invention also relates to a process for preparing an antibody directed against the p60 protein from listerias by immunizing an experimental animal with an immunogen and isolating the antibodies, characterized in that a polypeptide according to Figure 3 or an immunogenic conjugate which contains a polypeptide according to Figure 3 is used as the immunogen. In this context, immunogenic conjugates are preferred which contain a peptide having 7 to 24 amino acids selected from the polypeptide according to Figure 3, or which contain a peptide according to one of the formulae IVa-IVi, in which

X^3 and X^4 are each independently of one another hydrogen, an arbitrary amino acid or an arbitrary oligopeptide having up to 7 amino acids.

X^3ProValAlaProThrGlnX^4	IVa
X^3ThrGlnAlaThrThrProAlaX^4	IVb
X^3AlaIleLysGlnThrAlaAsnThrAlaX^4	IVc
X^3GlnGlnThrAlaProLysAlaProThrX^4	IVd
X^3ValAsnAsnGluValAlaAlaAlaGluLysThrGluX^4	IVe
X^3ThrProValLysGlnGluValLysX^4	IVf
X^3ValLysGlnProThrThrGlnGlnThrAlaProx^4	IVg
X^3IleLysGlnProThrLysThrValAlaProX^4	IVh
X^3GlnGlnThrThrThrLysAlaProThrX^4	IVi

Especially preferred are peptides having a sequence according to one of the figures 2a-i or 5a-d.

The invention further relates to the use of a primer, which contains a component sequence according to one of the formulae Ia-Ih or preferably a sequence according to one of the formulae IIa-IIh or an affiliated complementary sequence, for detecting bacteria of the genus *Listeria*.

The invention also relates to processes for detecting bacteria of the genus *Listeria* by means of a primer which contains a component sequence according to one of the formulae Ia-Ih or preferably a sequence according to one of the formulae IIa-IIh or an affiliated complementary sequence.

The invention further relates to the use of an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i or of the figures 5a-d, for detecting bacteria of the genus *Listeria*.

The invention also relates to processes for detecting bacteria of the genus *Listeria* by means of an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i, or to one of the Figures 5a-d.

The invention finally relates to test kits for detecting bacteria of the genus *Listeria*, in particular of the species *L. monocytogenes*, by means of the amplification of nucleic acids, for example by means of the polymerase chain reaction, which contain a primer having a component sequence according to one of the formulae Ia-Ih or preferably having a sequence according to one of the formulae IIa-IIh, or an affiliated complementary sequence.

The invention furthermore relates to test kits for the immunological detection of bacteria of the species *Listeria monocytogenes*, in which an antibody which is directed against an epitope from the polypeptide sequence according to Figure 3, or which is directed against one of the epitopes having an amino acid sequence according to one of the Figures 2a-i, is contained, as well as to test kits for the immunological detection of bacteria of the species *Listeria innocua*, in which an antibody, which is directed against an epitope having an amino acid sequence according to one of the Figures 5a-d is contained.

Brief Description of the Drawings

Various other objects, features and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawing, in which like reference characters designate the same or similar parts throughout the several views, and wherein:

Figure 1 shows the result of the electrophoretic separation of amplification products; the experimental details are presented in Example 8.

Figures 2a-i show the amino acid sequences of the particularly preferred immunogenic peptides selected from the sequence of the p60 protein from *Listeria monocytogenes*.

Figure 3 shows the amino acid sequence of the polypeptide selected from the sequence of the p60 protein from *Listeria monocytogenes*, whose epitopes are suitable for the immunological detection of bacteria of the genus *Listeria*.

Figure 4 shows, for comparative purposes, the amino acid sequence of the p60 protein from *Listeria monocytogenes*, which is presented in two component Figures a and b.

Figures 5a-d show the amino acid sequences of the particularly preferred immunogenic peptides selected from the sequence of protein p60 from *Listeria innocua*.

The invention is described below in more detail. In this context, the details of biochemical, immunological and molecular biological processes, which are known to the person skilled in the art and whose details are described in the literature, are presumed. In these processes, use can also be made of variations which are known per se but which are not described in detail here.

The oligonucleotides according to the invention described by the formulae Ia-Ih and IIa-IIh are suitable as primers for nucleic acid amplification methods, and thus for the specific detection of bacteria of the genus *Listeria*. Their sequences are presented in the customary manner, i.e., written from the 5' end to the 3' end. Depending on the requirements of the amplification system being used on any particular occasion, either deoxyribonucleotides or ribonucleotides having the sequences according to the invention are employed. In the latter case, the thymidine moieties are on each occasion replaced by uridine moieties. It is further known to the person skilled in the art that frequently the exchange of one or of a few bases in a nucleic acid sequence does not alter its biological properties. For this reason, the nucleotide sequences according to the invention also comprise those which are derived by base exchange from the sequences Ia-Ih and IIa-IIh, and which biologically show the same effect as the respective primer having the original sequence. Since normally one primer should in each case react with one of the DNA strands, one of the primers is employed in the complementary sequence. The complementary sequence is obtained in a known manner according to the rules for base pairing.

Based on the respective sequence, the oligonucleotides according to the invention can be synthesized by processes known to the person skilled in the art, for example by the phosphotriester or the phosphoamidite method. The phosphoamidite method is preferably employed, in particular using mechanized synthesizers. The method is described in Tetrahedron Lett. (1981) 22:1859-1862. Further details of synthetic processes of this nature are described, for example, in Winnacker, E.L. (1985) Gene und Klone [Genes and Clones], page 44-61 (VCH-Verlagsgesellschaft mbH, Weinheim).

The primers according to the invention are suitable for DNA amplification, for example using the polymerase chain reaction (PCR). For this purpose, the DNA is first dissociated into the single strands by heating. Two primers are used which in each case

hybridize with the homologous DNA segment on one of the DNA strands in each case. The genome segment which lies between these two primers is amplified. The primers attached to the DNA represent the starting points for the amplification. A polymerase, preferably *Taq* DNA polymerase, subsequently completes, in the presence of the four nucleotide triphosphates, the second strand corresponding to the sequence of the original DNA. Subsequently, the double strands which have arisen are dissociated once again into the single strands by heating. This amplification cycle can be repeated a number of times. After a sufficient number of amplification cycles, the amplified nucleic acid can be detected by means of known methods. For this purpose, the DNA can be separated by means of electrophoresis, and subsequently stained with ethidium bromide, and finally detected by fluorescence using UV excitation. Detection using DNA hybridization is also possible. The details of suitable amplification and detection methods are also described in review articles, e.g., Innis et al. (eds.), PCR Protocols (Academic Press, Inc., Harcourt Brace Jovanovich, Publishers). Other nucleic acid amplification processes in which the primers according to the invention can be used are also known from the literature. These include the ligase chain reaction, described by Bond, S. et al. ((1990), pp. 425-434, Raven Press (New York, NY/USA)).

The selection of the primers according to the preferred formulae IIa-IIh determines the position of the start points on the *iap* gene, and thus the specificity of the detection reaction: thus, combinations of primers selected from the sequence of the *iap* gene proved to be unspecific, and consequently unsuitable for detecting listerias by means of DNA amplification (in this connection see, for example, column F in Table 1). However, other selected combinations proved to be specific for the genus *Listeria*, others for groups of *Listeria* species, and others again for individual *Listeria* species. Altogether, therefore, the selection and the composition of the primers is critical. The selection of one of the two primers is always particularly critical, while the second primer can be more easily varied without significantly altering the specificity of the detection reaction. Consequently, according to the teaching of the present invention, for this second primer, a sequence can perfectly well be chosen which does not correspond to one of the formulae Ia-Ih or IIa-IIh.

According to the invention, at least one of the primers is selected from the formulae Ia-Ih or preferably from the formulae IIa-IIh. As already explained, the second primer has substantially less influence than the first primer on the specificity of the amplification reaction. However, combinations are preferred in which both primers are selected from the formulae Ia-Ih or IIa-IIh. Examples of preferred combinations of this nature are (typical results are summarized in Table 1):

a) When using a combination of a primer according to formula IIc with a primer with the complementary sequence according to formula IIb, only the DNA of listerias is amplified, and not the DNA of other types of bacteria (see column D in Table 1).

b) When using a combination of a primer according to formula IId with a primer with the complementary sequence according to formula IIb, only the DNA of *L. monocytogenes* is amplified, and not the DNA of other listerias or other bacteria (see column B in Table 1).

c) When using a combination of a primer according to formula IIf with a primer with the complementary sequence according to formula IIb, only the DNA of particular *Listeria* species is amplified, namely that of *L. seeligeri, L. welshimeri* and *L. ivanovii*, exclusively. This consequently permits group-specific detection (see column E in Table 1).

d) Another example of group-specific detection consists in the use of a combination of a primer according to formula IIh with a primer with the complementary sequence according to formula IIb: only the DNA of *L. grayi* and *L. murrayi* is amplified (see column G in Table 1).

e) Since the amplification products of different *Listeria* species exhibit varying molecular weights, bacteria of the genus *Listeria* can be differentiated by a combination of several primers (according to formulae IId, IIf, IIg and IIh) with the complementary sequence of formula IIb using one single polymerase reaction. Details of this further development of the polymerase technique are evident from Example 8 (see column H in Table 1, as well as Figure 1).

As already mentioned, it is also possible to detect the amplification products by nucleic acid hybridization. To do this, suitable nucleic acid fragments (nucleic acid probes) are added to the reaction mixture after the amplification. These nucleic acid probes possess a base sequence which is completely or partially complementary to the amplified gene segment. In addition, these probes are labelled for a detection reaction: they can contain radioactive isotopes, or carry fluorescent labels, or else be labelled by enzymes. Suitable labelling agents, methods for their introduction into the nucleic acid probe, and detection methods, are known to the person skilled in the art.

In particular, the amplification reaction can be designed specifically for the genus *Listeria* (as described in more detail above under a)) or for a group of *Listeria* species (as described above under c) and d)). By using nucleic acid probes which are in each case specific for one species, the presence of these species of *Listeria* can then be discerned in the reaction mixture. If the probes contain different labelling agents, different

species can also be detected side by side. This variation of the process consequently permits, in a similar manner to that described above under e), the detection of different *Listeria* species side by side.

The use of a nucleic acid probe, or of a mixture of different probes, which react with amplification products of all the *Listeria* species makes it possible to check the specificity of the amplification reaction or to prepare a unitary detection reagent for different *Listeria* species.

The peptides according to the invention, according to formulae IVa-IVi and according to Figure 2a-i or to figure 5a-d, can be incorporated into immunogenic conjugates. Using these conjugates, antibodies can be produced which make it possible specifically to detect bacteria of the genus *Listeria* using immunological methods.

The positions of the peptides according to the invention in the overall sequence of the p60 protein from *Listeria monocytogenes* are given below:

a) The sequence according to formula IIIa begins with proline at position 148 of the p60 sequence (Figure 4a); the peptides according to Figures 2a, 2e and 2f are also located in this region.

b) The sequence according to formula IIIb begins with threonine at position 178 of the p60 sequence (Figure 4a); the peptides according to Figures 2b and 2h are also located in this region.

c) The sequence according to formula IIIc begins with alanine at position 243 of the p60 sequence (Figure 4a); the peptides according to Figures 2c and 2i are also located in this region.

d) The sequence according to formula IIId begins with glutamine at position 292 of the p60 sequence (Figure 4b); the peptide according to Figure 2d is also located in this region.

e) The sequence according to formula IIIe begins with valine at position 71 of the p60 sequence (Figure 4a); the peptide according to Figure 2g is also located in this region.

The sequences of the peptides according to the invention which are shown in Figure 5a-d are derived from the total sequence of protein p60 from *Listeria innocua*; the same holds true for the partial sequences shown in formula IIIf-i and IVf-i.

It is known to the person skilled in the art that the exchange of one or of a few amino acids in a peptide frequently does not alter its biological properties. For this reason, the peptide sequences according to the invention also comprise those which are derived, by amino acid exchange, from the sequences according to Figures 2a-i, according to Figure 5a-d, or according to Figure 3, and which biologically show the same effect

as the respective peptides having the original sequence. One of skill in the art can routinely determine preferred exchanges in accordance with substitutions generally recognized as being preferred, e.g., according to the groups outlined in Dayhoff, M.O., Atlas of Protein Sequence and Structure, Vol. 5, p. 98 (1972), and updates thereof.

The selection of the peptides according to the invention proves to be critical. For example, when a particular peptide,

ThrAsnThrAsnThrAsnThrAsnThrAsnThrAsn

which is encoded by a gene segment around nucleotide 1390 which is specific for *L. monocytogenes*, was selected for the production of antibodies, none of the antisera, surprisingly, showed a reaction with the p60 protein.

Based on the sequence of the amino acids, the peptides can be synthesized by processes which are known to the person skilled in the art, for example by the t_{boc} or by the f_{moc} (tert-butyloxycarbonyl, or 9-fluorenylmethyloxycarbonyl) processes. Details of these processes are described, for example, in J. Am. Chem. Soc. 85, 2149-2154 (1963) and in Synthetic Polypeptides as Antigens (van Regenmortel et al. (eds.), Elsevier 1988 (volume 19 of the series Laboratory Techniques in Biochemistry and Molecular Biology). The f_{moc} process is preferred, in particular mechanized process variations thereof. Details of the process, as well as suitable amino acid protective groups, are known to the person skilled in the art.

Peptides are generally not suitable for producing antibodies. However, if peptides are coupled to high-molecular weight carrier substances, immunogenic conjugates are formed. The peptides according to the invention can be conjugated with known carrier substances. Among these are polyethylene glycols, serum albumins, KLH (keyhole limpet hemocyanin), ovalbumin, glucose dehydrogenase from *Bacillus megaterium* and PPD (purified protein derivative of tuberculin). Preferred carrier substances are KLH and glucose dehydrogenase from *B. megaterium*.

Besides this, bridging compounds (linkers) are frequently employed as well. These are low-molecular weight organic compounds having at least two linkable functional groups. Suitable compounds are known to the person skilled in the art; among these are, for example, 1,2-diaminoethane, succinic acid, β-alanine, 1,6-diaminohexane, 6-aminocaproic acid, adipic acid and cysteine. Cysteine is preferably employed as the linker, with this amino acid residue being incorporated during the synthesis of the peptide. Linkers which contain both an amino and a carboxyl function (e.g., β-alanine, 6-aminocaproic acid or cysteine), can be linked either at the C-terminus or at the N-terminus of the peptide. m-Maleimidobenzoic acid N-hydroxysuccinimide ester

(MBS) is preferably employed for preparing the bonds between the peptide and the carrier substance.

The said immunogenic conjugates serve to produce antibodies in experimental animals according to known processes. Usually, mammals are used for this purpose, for example sheep, goats, rabbits or mice. Rabbits are preferred for producing polyclonal antibodies. However, it is also possible to produce monoclonal antibodies using the immunogenic conjugates according to the invention.

Details of the immunological processes are known to the person skilled in the art. In addition, instructions for carrying out these processes are readily available in the literature; the following may be mentioned by way of example:

- Antibodies, E. Harlow and D. Lane, Cold Spring Harbor (1988)
- Woodard, L.F. and Jasman, R.L. (1985) Vaccine 3, 137-144
- Woodard, L.F. (1989) Laboratory Animal Sci. 39, 222-225
- Handbook of Experimental Immunology, Weir, D.M. et al. eds. (1986): Blackwell Scientific Publications, Oxford, GB.

Among these processes are, for example, the conjugation and immunization processes, as well the preparation and purification of antibodies, and also immunological detection processes. The immunological detection processes in which antibodies according to the invention can be used include preferably agglutination processes, immunometric detection processes, the immunoblot processes and in particular the sandwich ELISA processes.

According to the invention, the concept antibodies embraces both immunoglobulins and antisera. It is furthermore known to the person skilled in the art that, instead of a single antibody which is directed against a single epitope, a mixture of different antibodies of differing specificity may frequently be used. This results in advantages, in particular with regard to the sensitivity of detection. This applies in particular to monoclonal antibodies, but also to other antibodies, which are in each case directed against one epitope. Correspondingly, it can be advantageous to combine a plurality of antibodies which are directed against different peptide structures according to the formulae IIIa-IIIi or according to one of the Figures 3, 2a-i or 5a-d, for the use according to the invention and/or the processes according to the invention.

Details regarding the preparation of the primers and peptides according to the invention, as well as of their use, are evident from the following examples. The person skilled in the art will elicit further methodological details from the cited literature. The examples are intended to illustrate the subject of the invention, and do not represent any limitation of the invention.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight.

The entire disclosure of all applications, patents and publications, cited above and below, and of corresponding German applications P 42 19 111.4 and P 42 39 567.4, are hereby incorporated by reference.

EXAMPLES

Example 1: Preparation of the primer according to formula IIa

The primer according to formula IIa is prepared by the phosphoamidite method using the 380A DNA synthesizer from Applied Biosystems. The essential features of the method are described in Tetrahedron Lett. (1981) 22:1859-1862. Further details can be found in the literature supplied by the instrument manufacturer.

The primers according to formula IIc, IId, IIf, IIg and IIh are prepared in a corresponding manner. The primers according to formula IIb and IIe are prepared in the respective complementary sequence

(IIb: TTGGCTTCGGTCGCGTAGAATTCATA;

IIe: GCACTTGAATTGCTGTTATTG).

Example 2: Performance of the PCR reaction for the species-specific detection of *L. monocytogenes*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, primers according to formula IId and IIe (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C.

Subsequently, the reaction mixture is maintained at a temperature of 55°C for 30 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on a polyacrylamide gel (6%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

PCR products are only observed (see column A in Table 1) if DNA or cells from *L. monocytogenes* are present in the sample.

Example 3: Performance of the PCR reaction for the species-specific detection of *L. monocytogenes*

The process described in Example 2 is repeated using primers according to formula IId and IIb (see Example 1) instead of the primers according to formula IId and IIe. In

this case, too, PCR products are only observed if DNA or cells from *L. monocytogenes* are present in the sample (see column B in Table 1).

Table 1: Specificity of the polymerase chain reaction using different primers corresponding to formula IIa-IIh

Combination:	A	B	C	D	E	F	G	H
Primer 1:	IId	IId	IIg	IIc	IIf	IIa	IIh	M[3]
Primer 2:[1]	IIe	IIb	IIb	IIb	IIb	IIb	IIb	IIb
Reaction investigated								
Bacteria:								
L. monocytogenes								
serovar 1/2a EDG	+	+	-	+	-	+	-	+
serovar 1/2a Mack.[2]	+	+	-	+	-	+	-	+
serovar 1/2b	+	+	-	+	-	+	-	+
serovar 1/2c	+	+	-	+	-	+	-	+
serovar 3a	+	+	-	+	-	+	-	+
serovar 3b	+	+	-	+	-	+	-	+
serovar 3c	+	+	-	+	-	+	-	+
serovar 4a	+	+	-	+	-	+	-	+
serovar 4ab	+	+	-	+	-	+	-	+
serovar 4b	+	+	-	+	-	+	-	+
serovar 4c	+	+	-	+	-	+	-	+
serovar 4d	+	+	-	+	-	+	-	+
serovar 4e	+	+	-	+	-	+	-	+
serovar 7	+	+	-	+	-	+	-	+
L. ivanovii	-	-	-	+	+	+	-	+
L. seeligeri	-	-	-	+	+	+	-	+
L. innocua								
serovar 6a	-	-	+	+	-	+	-	+
serovar 6b	-	-	+	+	-	+	-	+
serovar 4ab	-	-	+	+	-	+	-	+
L. welshimeri	-	-	-	+	+	+	-	+
L. murrayi	-	-	-	+	-	+	+	+
L. grayi	-	-	-	+	-	+	+	+
Enterococcus faecalis	-	-	-	-	-	+	-	-
Bacillus cereus	-	-	-	-	-	+	-	-
Micrococcus flavus	-	-	-	-	-	+	-	-

Legend:
+ PCR product detected
- no PCR product detectable
1) Complementary sequence
2) Mack.: Mackaness strain
3) M: Mixture of primers according to formula IId, IIf, IIg and IIh;
Amplification products can be differentiated on the basis of the molecular weight.

Example 4: Performance of the PCR reaction for the genus-specific detection of bacteria of the genus *Listeria*

A sample of bacteria containing about 1 µg of DNA, is suspended in 50 µl water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIc and IIb (see Example 1; in each case 0.4 µg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 µmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 µl). The first denaturation step lasts for 3 minutes at 9°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 30 seconds (annealing phase), and at a temperature of 72°C for 2 minutes (elongation phase). The subsequent denaturation steps (at 94°C) each last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

In this case, PCR products are observed if DNA or cells from bacteria of the genus *Listeria* are present in the sample (see column D in Table 1).

Example 5: Performance of the PCR reaction for the group-specific detection of *listerias*

A sample of bacteria containing about 1 µg of DNA, is suspended in 50 µl water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIf and IIb (see Example 1; in each case 0.4 µg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 µmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 µl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 58°C for 45 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

In this case, PCR products are observed only if DNA or cells of bacteria from the group *L. ivanovii, L. seeligeri* and *L. welshimeri* are present in the sample (see column E in Table 1).

Example 6: Performance of the PCR reaction for the species-specific detection of *L. innocua*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIg and IIb (see Example 1; in each case 0.4 μg), and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 62°C for 60 seconds (annealing phase), and at a temperature of 72°C for 45 seconds (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

PCR products are only observed if DNA or cells of *L. innocua* are present in the sample (see column C in Table 1).

Example 7: Performance of the PCR reaction for the group-specific detection of *listerias*

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of water and heated at 110°C for 5 minutes. Subsequently, primers according to formula IIh and IIb (see Example 1; in each case 0.4 μg). and 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP, are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 45 seconds (annealing phase), and at a temperature of 72°C for 45 seconds (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on an agarose gel (1%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water) and visualized by irradiation with UV light (260 nm).

In this case, PCR products are only observed if DNA or cells of bacteria from the group *L. grayi* and *L. murrayi* are present in the sample (see column G in Table 1).

Example 8: **Performance of a combined PCR reaction for the species-specific detection of *L. monocytogenes* and of *L. innocua* and for the group-specific detection of the groups *L. ivanovii/L. seeligeri/L. welshimeri* and *L. grayi/L. murrayi***

A sample of bacteria containing about 1 μg of DNA, is suspended in 50 μl of buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl) and heated at 110°C for 5 minutes. Subsequently, a mixture of primers according to formula IId, IIf, IIg, IIh and IIb (see Example 1; in each case 0.4 μg), as well as 2.5 U of *Taq* polymerase (from Pharmacia), dissolved in reaction buffer (10 mM Tris-HCl pH 8.5; 1.5 mM $MgCl_2$ and 50 mM KCl), and in each case 200 μmol of dGTP, dATP, dTTP and dCTP, are added (total reaction volume 100 μl). The first denaturation step lasts for 3 minutes at 94°C. Subsequently, the reaction mixture is maintained at a temperature of 56°C for 45 seconds (annealing phase), and at a temperature of 72°C for one minute (elongation phase). The subsequent denaturation steps (at 94°C) last for 45 seconds. After 30 reaction cycles, a concluding elongation step (at 72°C), of 5-minute duration, is carried out.

The PCR products are separated on a polyacrylamide gel (4%) in a running buffer of Tris-borate (in each case 50 mM) containing EDTA (2.5 mM). In addition, a nucleic acid mixture (for example the product resulting from cleavage of *Spp*1 phage DNA by the restriction endonuclease *Eco*RI) is included as a molecular weight standard. Subsequently, the separated PCR products are stained by staining with ethidium bromide (0.1 mg/ml in water), and visualized by irradiation with UV light (260 nm).

The presence of DNA or cells of bacteria from the species *L. monocytogenes*, from the species *L. innocua*, from the group *L. ivanovii/L. seeligeri/L. welshimeri* or from the group *L. grayi/L. murrayi* can be differentiated on the basis of the different molecular weights (see column H in Table 1, as well as Figure 1).

Example 9: **Synthesis of the peptide**

CysGlnGlnGlnThrAlaProLysAlaProThrGlu

The f_{moc} process (9-fluorenylmethyloxycarbonyl protective group) is used for the synthesis of the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu. This peptide cor-

responds to a peptide of the formula IVd with an additional N-terminal cysteine residue as linker. A peptide synthesizer from Applied Biosystems is used for the synthesis; the process parameters are contained in the instrument documentation.

A polymeric support with 4-(2'4'-dimethoxyphenylaminomethyl)phenoxy groups serves as the solid phase. The amino acids are employed as α-N-f_{moc} derivatives. Any reactive side groups contained in the amino acids are masked by additional protective groups which may be eliminated by hydrolysis with trifluoroacetic acid. The peptide bonds are produced by activating the carboxyl groups with diisopropylcarbodiimide. The order in which the amino acid derivatives are put in is determined by the desired sequence.

In the first step of the synthesis cycle, the amino group on the solid phase, i.e., in the first cycle the amino groups of the 4-(2'4'-dimethoxyphenylaminomethyl)phenoxy residue of the support, reacts with the carboxyl group of the incoming amino acid, which is employed as the α-N-f_{moc} derivative, where appropriate with protected side chains, and which is activated by diisopropylcarbodiimide, as does the α-amino group of the last amino acid to be attached in the following cycles. Amino acid derivatives which have not reacted are washed out with dimethylformamide. Subsequently, the f_{moc} group is eliminated by treating with 20% (V/V) piperidine in dimethylformamide. The rest of the protective groups remain unaltered during this reaction. Following the removal of the α-N-protective group, the next reaction cycle can begin. Once the last amino acid corresponding to the envisaged sequence has been added, the protective groups of the side chains and the bond with the support resin are cleaved by acid hydrolysis with trifluoroacetic acid. The peptide is subsequently purified by high pressure liquid chromatography.

The remaining peptides with the sequences according to the invention are also synthesized in accordance with the procedure described above.

Example 10: Conjugation of the peptide

CysGlnGlnGlnThrAlaProLysAlaProThrGlu with glucose dehydrogenase

a) Derivatization of the glucose dehydrogenase: 30 mg of glucose dehydrogenase from *Bacillus megaterium* from Merck (Art. No. 13732) are dissolved in 4 ml of sodium phosphate buffer (50 mM; pH 8.0). 6.78 mg of N-y-maleimidobutyryloxysuccinimide (from Calbiochem), dissolved in 50 μl of dimethyl sulfoxide, are added to 2.4 ml of this solution, and the mixture is left to stand at room temperature for 30 minutes. Subsequently, the excess N-y-maleimidobutyryloxysuccinimide is separated off chromatographically by gel filtration on PD-10 (from Pharmacia). Following the chromatography,

3.5 ml of a solution of the activated carrier protein are obtained, having a concentration of 4.5 mg/ml.

b) Coupling with the peptide: 5.2 mg of the peptide, prepared according to Example 9 and dissolved in 1 ml of sodium phosphate buffer (50 mM; pH 7.0), are added to 1.1 ml of the solution from the above step and the mixture is left to stand at room temperature for 3 hours. Subsequently, the peptide which has not been bound is separated off chromatographically by gel filtration on PD-10 (from Pharmacia). Following the chromatography, 3.5 ml of a solution of the conjugate are obtained, having a concentration of 2.3 mg/ml.

Conjugates with other peptides corresponding to the present invention are also prepared in accordance with the procedure described above.

Example 11: Production of polyclonal antibodies against the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu

Two rabbits are in each case injected intramuscularly with an emulsion consisting of 0.18 ml of conjugate from Example 10, 0.07 ml of phosphate-buffered saline and 0.25 ml of an oil adjuvant (MISA 50, from Seppic, France). Booster injections of the same quantities are given three, five and seven weeks after the initial injection. One week after the last injection, the animals are killed and exsanguinated. After the blood has coagulated, the antiserum is obtained by centrifugation and sodium azide is added to give a final concentration of 0.02%. The antiserum is stored frozen at -20°C.

Example 12: Production of monoclonal antibodies against the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu

Two mice are in each case injected subcutaneously with an emulsion consisting of 0.1 ml of conjugate from Example 10 and 0.1 ml oil adjuvant (MISA 50, from Seppic, France). Booster injections of the same amounts are given two, four and six weeks after the initial injection. Three days after the last injection, the animals are killed and the spleen is isolated. The cells from the spleen are isolated by customary processes and fused with a permanent murine cell line. Cell lines which form antibodies again the peptide CysGlnGlnGlnThrAlaProLysAlaProThrGlu are selected from the fusion products.

Example 13: Immunological detection of *L. monocytogenes*

a) Pre-culture and centrifugation of the bacteria: 10 ml of CASO broth are inoculated with material from several colonies of *L. monocytogenes* and incubated at 30°C

overnight. Subsequently, 1 ml of the culture is withdrawn in each case. The bacterial cells are removed by centrifugation (13000 rpm).

b) Identification reaction: In each case 300 µl of the supernatants from the previous step are pipetted into the wells of a microtiter plate and incubated at 4°C overnight. Subsequently, each well is washed three times with 100 µl of washing solution (9 g/l NaCl and 0.05% Tween 20 in water) on each occasion. 100 µl of antiserum prepared according to Example 11 are now pipetted into each well and the plate is incubated at room temperature for one hour. Each well is once again washed three times with 100 µl of washing solution on each occasion. 100 µl of anti-rabbit antibody solution labelled with alkaline phosphatase (Art. No. A 8025, from Sigma) are then pipetted into each of the wells, and the plate is incubated at room temperature for 30 minutes. Each of the wells is washed once again with 100 µl of washing solution and the bound, enzyme-labelled antibody is subsequently detected. To do this, 200 µl of a buffer solution containing substrate is added to each well, and the plate is incubated at room temperature for 30 minutes. The reaction is stopped by the addition of 100 µl of 2N NaOH solution (Art. No. 9136, from Merck) to each well, and the reaction product is rendered visible. A yellow-orange coloration indicates the presence of *L. monocytogenes*.

Example 14: Specific detection of *L. monocytogenes* using immunoblotting

Bacteria are pre-cultured as described in Example 13a), and the cells are centrifuged off. The cells which have been centrifuged off are taken up and suspended in 1 ml of phosphate-buffered saline. 2 µl of this suspension are pipetted on to a nitrocellulose membrane (Hybond C, 0.45 µm, Art. No. RPN 283 C, from Amersham). Once the solution has dried in, the membrane is treated at room temperature for one hour with a solution of bovine serum albumin (10 g/l) in phosphate-buffered saline. A dilution (1:200) of the antiserum obtained in Example 11 is prepared using a solution of bovine serum albumin (10 g/l) and Tween 20 (0.5 g/l) in phosphate-buffered saline (antibody solution A), as is a further dilution (1:500) of peroxidase-labelled anti-rabbit antibody (anti-rabbit IgG, Art. No. 68-397; from ICN Immunobiologicals) using the same diluent (HRP-antibody solution). The membrane is incubated at room temperature for one hour with antibody solution A. and subsequently washed three times with phosphate-buffered saline containing 0.05% Tween 20. In order to detect the antibody binding, the membrane is subsequently incubated at room temperature for one hour with HRP-antibody solution and in each case washed three times with a) Tween 20 (0.5 g/l) in phosphate-buffered saline, b) phosphate-buffered saline and c) Tris buffer (50 mM; pH 7.4; containing 200 mM NaCl). For the color reaction, a solution of 4-chloro-1-naphthol

(3 mg/ml in methanol) is diluted with five volumes of Tris buffer (50 mM; pH 7.4; containing 200 mM NaCl), and hydrogen peroxide is added (final concentration 0.1 g/l). The membrane is incubated in this substrate solution. A blue-black coloration indicates *L. monocytogenes*.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

3. An isolated peptide selected from the formulae (IVa)-(IVi)

X^3ProValAlaProThrGlnX^4	(IVa)
X^3ThrGlnAlaThrThrProAlaX^4	(IVb)
X^3AlaIleLysGlnThrAlaAsnThrAlaX^4	(IVc)
X^3GlnGlnThrAlaProLysAlaProThrX^4	(IVd)
X^3ValAsnAsnGluValAlaAlaAlaGluLysThrGluX^4	(IVe)
X^3ThrProValValLysGlnGluValLysX^4	(IVf)
X^3VaLysGlnProThrThrGlnGlnThrAlaProX^4	(IVg)
X^3IleLysGlnProThrLysThrValAlaProX^4	(IVh)
X^3GlnGlnThrThrThrLysAlaProThrX^4	(IVi)

wherein
X^3 and X^4 are each, independently, hydrogen or 1-7 additional amino acids.

4. An isolated peptide of claim 3, wherein said peptide is useful as an epitope for preparing or specifically binding to an antibody to p60 protein.

5. A peptide of claim 3, selected from the sequences of Figures 2a-i.

6. A peptide of claim 3, selected from the sequences of Figures 5a-d.

7. A method of preparing an immunogenic conjugate, wherein one portion of the conjugate comprises a peptide of claim 3.

8. A process for preparing an antibody capable of specifically binding the p60 protein from listerias, comprising

immunizing an experimental animal with an immunogen, wherein said immunogen is a polypeptide of Figure 3 or an immunogenic conjugate which comprises a peptide having a sequence selected from the polypeptide sequence of Figure 3, and

isolating the thus-produced antibody.

9. A process of claim 8, wherein the immunogenic conjugate comprises a 7-24 amino acid peptide having a sequence selected from the polypeptide sequence according to Figure 3.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule selected from one of the formulae (Va)-(Vh), wherein

X^1 and X^2 are each, independently, hydrogen or 1 – 20 additional nucleotides,

X^1AATATGAAAAAAGCX^2	(Va),
X^1GCTTCGGTCGCGTAX^2	(Vb),
X^1ACAGCTGGGATTGCX^2	(Vc),
X^1ACTGCTAACACAGCTX^2	(Vd),
X^1TAACAGCAATTCAAGX^2	(Ve),
X^1CTGAGGTAGCGAGCX^2	(Vf),
X^1AGCACTCCAGTTGTTAX^2	(Vg), or
X^1GCAGTTTCTAAACCTX^2	(Vh),

or a DNA molecule complementary to said sequence of formulae (Va)-(Vh), wherein said sequence is useful as a primer for amplifying an *iap* (invasion-associated protein) gene.

2. An isolated DNA molecule of claim 1, selected from

ATGAATATGAAAAAAGCAAC	(IIa),
TTGGCTTCGGTCGCGTATAA	(IIb),
GCTACAGCTGGGATTGCGGT	(IIc),
CAAACTGCTAACACAGCTACT	(IId),
CAATAACAGCAATTCAAGTGC	(IIe),
TAACTGAGGTAGCGAGCGAA	(IIf),
ACTAGCACTCCAGTTGTTAAAC	(IIg), or
CCAGCAGTTTCTAAACCTGCT	(IIh),

or a DNA molecule complementary to said sequence of formulae (IIa)-(IIh).

10. A process of claim 8, wherein the immunogenic conjugate comprises a peptide according to one of the formulae (IVa)-(IVi) of Figure 3.

11. A method of detecting the presence of a bacteria of the genus *Listeria* in a sample, comprising hybridizing DNA from the sample with a DNA molecule of claim 1.

12. A method of detecting the presence of a bacteria of the genus *Listeria* in a sample by means of gene amplification, wherein a primer of claim 1 is used.

13. A test kit for detecting bacteria of the genus *Listeria* by means of a polymerase chain reaction assay comprising, as a DNA primer, a DNA molecule of claim 1.

14. A test kit of claim 13 for detecting bacteria of the species *Listeria monocytogenes*.

15. An isolated antibody which specifically binds to the p60 protein from pathogenic listerias, wherein said antibody binds an epitope from the sequence of the polypeptide of Figure 3.

16. An antibody of claim 15, wherein said antibody binds an epitope comprising a sequence of Figures 2a-i.

17. An isolated antibody which can be prepared by immunizing an experimental animal with a polypeptide according to Figure 3, or with an immunogenic conjugate which conjugate comprises a 7-24 amino acid peptide having a sequence selected from the polypeptide sequence of Figure 3, wherein said antibody specifically binds to the p60 protein from pathogenic listerias.

18. An isolated antibody which specifically binds a p60 protein from listerias, wherein said antibody binds an epitope which comprises a sequence of Figures 5a-d.

19. An antibody which can be prepared by immunizing an experimental animal with an immunogenic conjugate, wherein the conjugate comprises a peptide of Figures 5a-d.

20. A method of detecting bacteria of the genus *Listeria* in a sample, comprising binding a p60 protein with an antibody of claim 15.

21. A method of detecting bacteria of the genus *Listeria* in a sample by means of an immune reaction, wherein an antibody of claims 15 is used.

22. A test kit for detecting bacteria of the species *Listeria monocytogenes* by immunoassay, comprising an antibody of claim 15.

23. A test kit for detecting bacteria of the species *Listeria innocua* by immunoassay, wherein it contains an antibody of claim 18.

ABSTRACT OF THE DISCLOSURE

The invention relates to agents and processes for detecting bacteria of the genus *Listeria*, in particular *L. monocytogenes*. The agents according to the invention include primers whose sequence is selected from the *iap* gene of *L. monocytogenes*. In addition, the agents according to the invention include peptides whose sequence is selected from the p60 protein and which are suitable for producing specific antibodies for the immunological detection of *L. monocytogenes*.

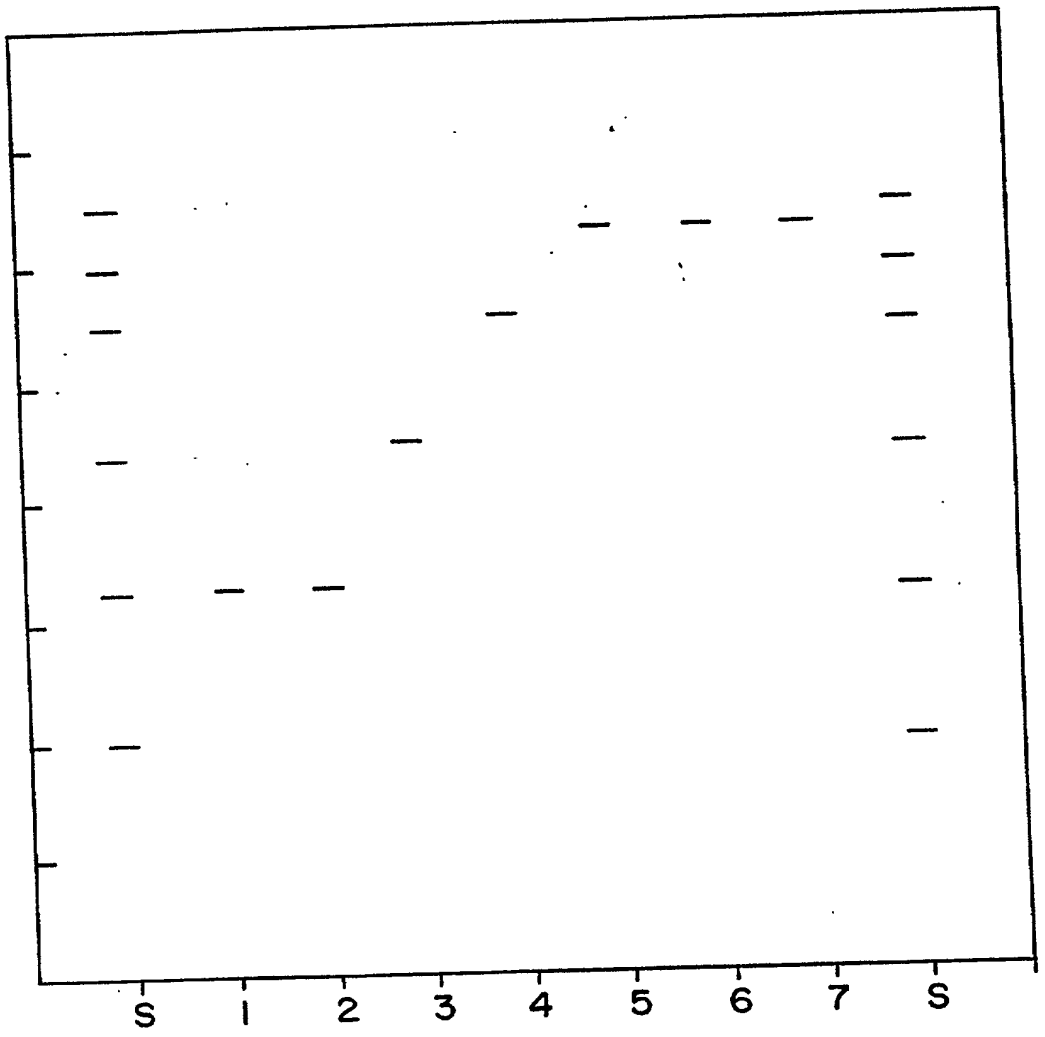

FIG. 1

Val Ser Thr Pro Val Ala Pro Thr Gln
1 5

FIG. 2a

Thr Thr Gln Ala Thr Thr Pro Ala Pro Lys Val Ala
1 5 10

FIG. 2b

Leu Ala Ile Lys Gln Thr Ala Asn Thr Ala Thr
1 5 10

FIG. 2c

Gln Gln Gln Thr Ala Pro Lys Ala Pro Thr Glu
1 5 10

FIG. 2d

Ser Thr Pro Val Ala Pro Thr Gln Glu Val Lys Lys
1 5 10

FIG. 2e

Pro Val Ala Pro Thr Gln Glu Val Lys Lys
1 5 10

FIG. 2f

Gln Val Asn Asn Glu Val Ala Ala Ala Glu Lys Thr Glu Lys
1 5 10

FIG. 2g

Glu Val Lys Gln Thr Thr Gln Ala Thr Thr Pro Ala
1 5 10

FIG. 2h

Ala Ile Lys Gln Thr Ala Asn Thr Ala Thr Pro Lys
1 5 10

FIG. 2i

```
Gln Val Asn Asn Glu Val Ala Ala Ala Glu Lys Thr Glu Lys Ser Val
 1               5                  10                  15

Ser Ala Thr Trp Leu Asn Val Arg Thr Gly Ala Gly Val Asp Asn Ser
            20                  25                  30

Ile Ile Thr Ser Ile Lys Gly Gly Thr Lys Val Thr Val Glu Thr Thr
        35                  40                  45

Glu Ser Asn Gly Trp His Lys Ile Thr Tyr Asn Asp Gly Lys Thr Gly
    50                  55                  60

Phe Val Asn Gly Lys Tyr Leu Thr Asp Lys Ala Val Ser Thr Pro Val
65                  70                  75                  80

Ala Pro Thr Gln Glu Val Lys Lys Glu Thr Thr Thr Gln Gln Ala Ala
                85                  90                  95

Pro Val Ala Glu Thr Lys Thr Glu Val Lys Gln Thr Thr Gln Ala Thr
            100                 105                 110

Thr Pro Ala Pro Lys Val Ala Glu Thr Lys Glu Thr Pro Val Ile Asp
        115                 120                 125

Gln Asn Ala Thr Thr His Ala Val Lys Ser Gly Asp Thr Ile Trp Ala
    130                 135                 140

Leu Ser Val Lys Tyr Gly Val Ser Val Gln Asp Ile Met Ser Trp Asn
145                 150                 155                 160

Asn Leu Ser Ser Ser Ser Ile Tyr Val Gly Gln Lys Leu Ala Ile Lys
                165                 170                 175

Gln Thr Ala Asn Thr Ala Thr Pro Lys Ala Glu Val Lys Thr Glu Ala
            180                 185                 190

Pro Ala Ala Glu Lys Gln Ala Ala Pro Val Val Lys Glu Asn Thr Asn
        195                 200                 205

Thr Asn Thr Ala Thr Thr Glu Lys Lys Glu Thr Ala Thr Gln Gln Gln
    210                 215                 220

Thr Ala Pro Lys Ala Pro Thr Glu
225                 230
```

FIG. 3

Met Asn Met Lys Lys Ala Thr Ile Ala Ala Thr Ala Gly Ile Ala Val
1 5 10 15

Thr Ala Phe Ala Ala Pro Thr Ile Ala Ser Ala Ser Thr Val Val Val
20 25 30

Glu Ala Gly Asp Thr Leu Trp Gly Ile Ala Gln Ser Lys Gly Thr Thr
35 40 45

Val Asp Ala Ile Lys Lys Ala Asn Asn Leu Thr Thr Asp Lys Ile Val
50 55 60

Pro Gly Gln Lys Leu Gln Val Asn Asn Glu Val Ala Ala Ala Glu Lys
65 70 75 80

Thr Glu Lys Ser Val Ser Ala Thr Trp Leu Asn Val Arg Thr Gly Ala
85 90 95

Gly Val Asp Asn Ser Ile Ile Thr Ser Ile Lys Gly Gly Thr Lys Val
100 105 110

Thr Val Glu Thr Thr Glu Ser Asn Gly Trp His Lys Ile Thr Tyr Asn
115 120 125

Asp Gly Lys Thr Gly Phe Val Asn Gly Lys Tyr Leu Thr Asp Lys Ala
130 135 140

Val Ser Thr Pro Val Ala Pro Thr Gln Glu Val Lys Lys Glu Thr Thr
145 150 155 160

Thr Gln Gln Ala Ala Pro Val Ala Glu Thr Lys Thr Glu Val Lys Gln
165 170 175

Thr Thr Gln Ala Thr Thr Pro Ala Pro Lys Val Ala Glu Thr Lys Glu
180 185 190

Thr Pro Val Ile Asp Gln Asn Ala Thr Thr His Ala Val Lys Ser Gly
195 200 205

Asp Thr Ile Trp Ala Leu Ser Val Lys Tyr Gly Val Ser Val Gln Asp
210 215 220

Ile Met Ser Trp Asn Asn Leu Ser Ser Ser Ser Ile Tyr Val Gly Gln
225 230 235 240

Lys Leu Ala Ile Lys Gln Thr Ala Asn Thr Ala Thr Pro Lys Ala Glu
245 250 255

FIG. 4a

Val Lys Thr Glu Ala Pro Ala Ala Glu Lys Gln Ala Ala Pro Val Val
260 265 270

Lys Glu Asn Thr Asn Thr Asn Thr Ala Thr Thr Glu Lys Lys Glu Thr
275 280 285

Ala Thr Gln Gln Gln Thr Ala Pro Lys Ala Pro Thr Glu Ala Ala Lys
290 295 300

Pro Ala Pro Ala Pro Ser Thr Asn Thr Asn Ala Asn Lys Thr Asn Thr
305 310 315 320

Asn Thr Asn Thr Asn Asn Thr Asn Thr Pro Ser Lys Asn Thr Asn Thr
325 330 335

Asn Ser Asn Thr Asn Thr Asn Thr Asn Ser Asn Thr Asn Ala Asn Gln
340 345 350

Gly Ser Ser Asn Asn Asn Ser Asn Ser Ser Ala Ser Ala Ile Ile Ala
355 360 365

Glu Ala Gln Lys His Leu Gly Lys Ala Tyr Ser Trp Gly Gly Asn Gly
370 375 380

Pro Thr Thr Phe Asp Cys Ser Gly Tyr Thr Lys Tyr Val Phe Ala Lys
385 390 395 400

Ala Gly Ile Ser Leu Pro Arg Thr Ser Gly Ala Gln Tyr Ala Ser Thr
405 410 415

Thr Arg Ile Ser Glu Ser Gln Ala Lys Pro Gly Asp Leu Val Phe Phe
420 425 430

Asp Tyr Gly Ser Gly Ile Ser His Val Gly Ile Tyr Val Gly Asn Gly
435 440 445

Gln Met Ile Asn Ala Gln Asp Asn Gly Val Lys Tyr Asp Asn Ile His
450 455 460

Gly Ser Gly Trp Gly Lys Tyr Leu Val Gly Phe Gly Arg Val
465 470 475

FIG. 4b

Ser Thr Pro Val Val Lys Gln Glu Val Lys Lys
1 5 10

FIG. 5a

Glu Val Lys Gln Pro Thr Thr Gln Gln Thr Ala Pro Ala
1 5 10

FIG. 5b

Ala Ile Lys Gln Pro Thr Lys Thr Val Ala Pro Lys
1 5 10

FIG. 5c

Glu Gln Gln Thr Thr Thr Lys Ala Pro Thr Gln
1 5 10

FIG. 5d

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
MERCK 1502

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PROCESSES AND AGENTS FOR DETECTING LISTERIAS

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. _____

on _____,

and was amended

on _____ (if applicable).

☐ was filed as PCT international application

Number _____

on _____,

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowlege the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

RIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Germany	P 42 39 567.4	25 November 1992	☒ YES ☐ NO
Germany	P 42 19 111.4	11 June 1992	☒ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO

Combined Declaration For Patent Application and Power of Attorney (Continued)

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: MERCK 1502

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED *(if any)*			

POWER OF ATTORNEY: As a named inventor, I hereby appoint I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E. J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Diana Hamlet-King (33,302); Richard J. Traverso (30,595); Richard E. Kurtz (33,936); John A. Sopp (33,103) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Send Correspondence to: MILLEN, WHITE, ZELANO AND BRANIGAN, P.C.
Arlington Courthouse Plaza I, Suite 1400
2200 Clarendon Boulevard
Arlington, Virginia 22201

Telephone No. 703-243-6333

Direct Telephone Calls to: Diana Hamlet-King

201	FULL NAME OF INVENTOR	FAMILY NAME: SCHUBERT	FIRST GIVEN NAME: Peter	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6100 Darmstadt	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
202	FULL NAME OF INVENTOR	FAMILY NAME: NEUMANN	FIRST GIVEN NAME: Siegfried	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6104 Seeheim-Jugenheim	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
203	FULL NAME OF INVENTOR	FAMILY NAME: PAWELZIK	FIRST GIVEN NAME: Martina	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-8000 München	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
204	FULL NAME OF INVENTOR	FAMILY NAME: LINXWEILER	FIRST GIVEN NAME: Winfried	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6114 Gross-Umstadt	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
205	FULL NAME OF INVENTOR	FAMILY NAME: BURGER	FIRST GIVEN NAME: Christa	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6100 Darmstadt	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
206	FULL NAME OF INVENTOR	FAMILY NAME: HOFMANN	FIRST GIVEN NAME: Gottfried	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6100 Darmstadt	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E. MERCK Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany

Combined Declaration For Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: Merck 1502

207	FULL NAME OF INVENTOR	FAMILY NAME: BUBERT	FIRST GIVEN NAME: Andreas	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-6708 Gerbrunn	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E.Merck Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
208	FULL NAME OF INVENTOR	FAMILY NAME: GOEBEL	FIRST GIVEN NAME: Werner	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: D-8707 Veitshöchheim	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E.Merck Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
209	FULL NAME OF INVENTOR	FAMILY NAME: KÖHLER	FIRST GIVEN NAME: Stefan	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: F-34980 St.Clément de Rivière	STATE OR FOREIGN COUNTRY: France	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: c/o E.Merck Postfach 4119	CITY: D-6100 Darmstadt	STATE & ZIP CODE/COUNTRY: Germany
210	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	STREET:	CITY:	STATE & ZIP CODE/COUNTRY:
211	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	STREET:	CITY:	STATE & ZIP CODE/COUNTRY:
212	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:
	POST OFFICE ADDRESS	STREET:	CITY:	STATE & ZIP CODE/COUNTRY:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201: Werner Goebel	DATE: June 1, 1993	SIGNATURE OF INVENTOR 207: Peter Schuster	DATE: June 1, 1993
SIGNATURE OF INVENTOR 202: Andreas Bubert	DATE: June 1, 1993	SIGNATURE OF INVENTOR 208: Winfried Weissenhorn	DATE: June 1, 1993
SIGNATURE OF INVENTOR 203: Stephan Köhler	DATE: June 1, 1993	SIGNATURE OF INVENTOR 209: Ala By	DATE: June 1, 1993
SIGNATURE OF INVENTOR 204: Martina Pawlik	DATE: June 1, 1993	SIGNATURE OF INVENTOR 210:	DATE:
SIGNATURE OF INVENTOR 205: Alfred Hofmann	DATE: June 1, 1993	SIGNATURE OF INVENTOR 211:	DATE:
SIGNATURE OF INVENTOR 206: Siegfried Neumann	DATE: June 1, 1993	SIGNATURE OF INVENTOR 212:	DATE: